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Cytokines, Transcription Factors, and the Initiation of T-Cell Development

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Multipotent blood progenitor cells migrate into the thymus and initiate the T-cell differentiation program. T-cell progenitor cells gradually acquire T-cell characteristics while shedding their multipotentiality for alternative fates. This process is supported by extracellular signaling molecules, including Notch ligands and cytokines, provided by the thymic microenvironment. T-cell development is associated with dynamic change of gene regulatory networks of transcription factors, which interact with these environmental signals. Together with Notch or pre-T-cell-receptor (TCR) signaling, cytokines always control proliferation, survival, and differentiation of early T cells, but little is known regarding their cross talk with transcription factors. However, recent results suggest ways that cytokines expressed in distinct intrathymic niches can specifically modulate key transcription factors. This review discusses how stage-specific roles of cytokines and transcription factors can jointly guide development of early T cells.

The thymus is the organ specialized to make T cells. T cells originate from hematopoietic stem and precursor cells in the bone marrow or fetal liver, which migrate to the thymus and acquire T-cell identity. Relatively small numbers of T-cell progenitors migrate into the thymus per day, but they respond to the new environment by undergoing multiple rounds of proliferation while initiating the T-cell differentiation program (Rothenberg 2000; Petrie and Zuniga-Pflucker 2007; Rothenberg et al. 2008; Love and Bhandoola 2011; Naito et al. 2011; Thompson and Zúñiga-Pflücker 2011; Rothenberg 2014; Yui and Rothenberg 2014). They then undergo T-cell lineage commitment, begin T-cell receptor (TCR) rearrangements, and thus generate αβTCR- or γδTCR-expressing T cells. The αβ T cells further diverge into different sublineages, such as CD4 T cells, CD8 T cells, natural killer T (NKT) cells and regulatory T (Treg) cells, ultimately to act as a "conductor" of the immune system "orchestra."

Thymocytes are divided into multiple phenotypically distinct stages that are defined by the expression of CD4, CD8, and other markers (Hayday and Pennington 2007; Rothenberg et al. 2008; Yang et al. 2010; Naito et al. 2011; Yui and Rothenberg 2014). T-cell development is initiated from the subpopulation that lacks the expression of both CD4 and CD8, thus called double-negative (DN) cells, which then become $CD4^+$ $CD8^+$ double-positive (DP) and subsequently differentiate into mature CD4 or CD8 single-positive (SP) cells. The earliest T-cell precursors in the thymus, called early thymic progenitor (ETP) or Kit-high double-negative 1 (KIT^{++} DN1; $CD44^+$ $CD25^-$), still harbor the potential to gain access to non-T

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alternative fates. These cells start expressing T-cell markers in the next stage, DN2a (KIT^{++} $CD44^{+}$ $CD25^{+}$), but commitment to the T-cell lineage occurs only at the following stage, DN2b (Kit^{+} $CD44^{+}$ $CD25^{+}$). Then in the DN3a (KIT^{-} $CD44^{-}$ $CD25^{+}$) stage, *TCRβ* gene rearrangement begins. This process enables some cells to express either a pre-TCR (TCRβ with invariant pre-TCRα) or a γδTCR. Pre-TCR-mediated signal transduction triggers transition of DN3a cells through DN3b into DN4 (Kit^{-} $CD44^{-}$ $CD25^{-}$), followed by progression to the DP stage. DP thymocytes undergo *TCRα* gene rearrangement and begin to express fully assembled αβTCR. Then, they are subjected to a selection process, which is known as positive selection, to identify cells that express TCR with potentially useful ligand specificities. Positively selected thymocytes are allowed to differentiate into either CD4 helper T cells or CD8 cytotoxic T cells, known as CD4/CD8-lineage choice.

The special feature of the thymic cortical environment is its dense presentation of Notch ligand, primarily Delta-like ligand 4 (DLL4) (Love and Bhandoola 2011). Very early in the ETP stage, T-cell precursors become not only influenced by Notch-DLL4 interaction but dependent on it for optimal growth and survival. NOTCH1 molecules on the surface of lymphoid precursors interact with DLL4 on thymic stromal cells, driving lymphoid precursors to initiate the T-cell-specific developmental program. Engagement of cell-surface NOTCH1 by environmental Notch ligands triggers the proteolytic release of intracellular NOTCH1, which travels to the nucleus to become a direct coactivator of DNA-bound recombining binding protein suppressor of hairless (RBPJ) and stimulates the expression of Notch target genes (Radtke et al. 2010). All of the events that establish the T-cell identity of precursors are driven directly or indirectly by Notch signaling (Schmitt and Zuniga-Pflucker 2002; Thompson and Zúñiga-Pflücker 2011).

THREE PHASES OF EARLY T-CELL DEVELOPMENT

Early T-cell precursor development can be divided usefully into three phases in which the first two depend on Notch signaling and the third depends on signals from the pre-TCR. The first Notch-dependent phase involves the expansion of uncommitted T-cell precursors. The second Notch-dependent phase establishes the competence of the cells to express and depend on TCR complexes. The third phase, much less Notch-dependent, expands cells with well-assembled pre-TCR complexes and prepares them for full immunological repertoire selection. These stages of differentiation are shown in Figure 1.

Phase 1

Under the influence of Notch signaling, the ETP cells that are derived from KIT^{hi} interleukin-7 receptor $(IL\text{-}7R)^{low}$ precursors develop into KIT^{hi} $IL\text{-}7R^{hi}$ DN2a cells before undergoing T-cell commitment. Cytokines made by the thymic stroma act as growth factors for them and support extensive proliferative expansion of precommitment precursors (Love and Bhandoola 2011; Yui and Rothenberg 2014). Notch signaling not only turns on canonical Notch target genes such as *Hes1,* but also initiates the expression of the crucial T-cell specification factor coding genes *Gata3* and *Tcf7* (encoding TCF1 protein). Even before the cells are committed, ETP and DN2a cells begin to express some T-cell-specific genes. Together with Notch signaling, GATA3 and TCF1 antagonize the progenitor-specific factors in these pro-T cells and regulate specification and commitment of T cells (Rosenbauer et al. 2006; Hosoya et al. 2009; Germar et al. 2011; Weber et al. 2011; De Obaldia et al. 2013b; Garcia-Ojeda et al. 2013; Scripture-Adams et al. 2014).

Phase 2

Transcriptome analysis of developing T cells shows that the commitment-linked transition from phase 1 to phase 2 is marked by a large number of positive and negative gene regulation changes (Zhang et al. 2012). One element of these transcriptional changes is the activation of *Bcl11b* (Ikawa et al. 2010; Li et al. 2010a,b). BCL11B is a six zinc-finger transcriptional repressor that is turned on in the late DN2a stage by Notch signaling, TCF1, RUNX1, and

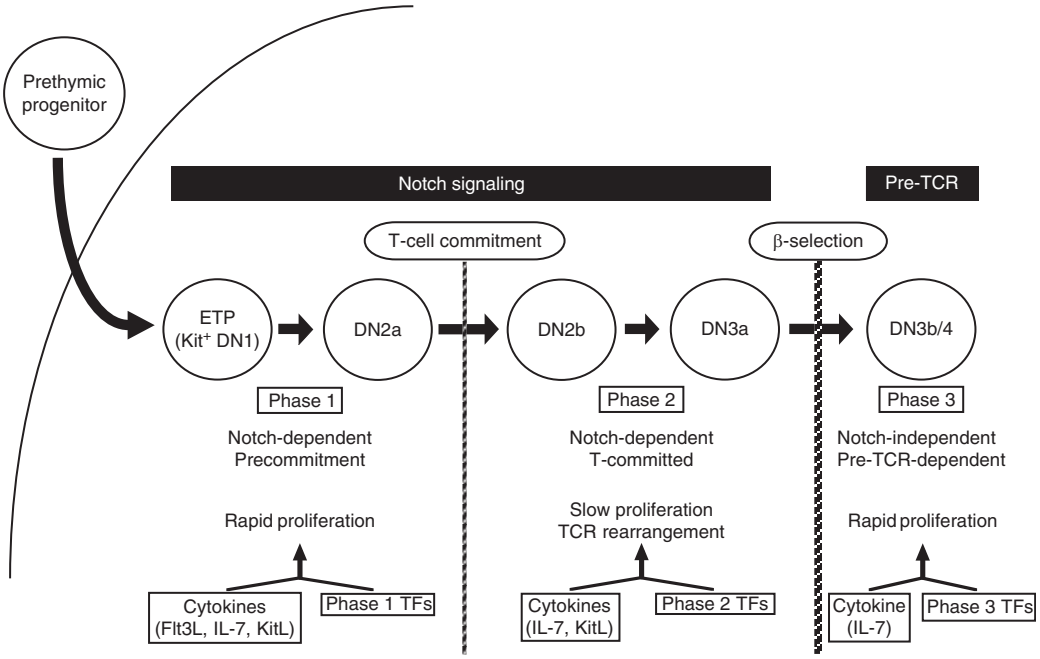

Figure 1. Roles of cytokines and transcription factors (TFs) in three phases of early T-cell development. Prethymic progenitor cells migrate into the thymus and begin T-cell differentiation program under the influence of Notch signaling. The earliest T-cell precursors in the thymus are called early thymic progenitor (ETP) or KIT-high double-negative 1 (KIT^{++} DN1; $CD44^{+}$ $CD25^{-}$) and they transit through DN2a, DN2b, DN3a, and DN3b/4 stages, followed by progression to DP stage. Early T-cell development can be divided into three phases based on the status of T-lineage commitment and Notch-dependency. The proliferation rate of the cells dynamically changes during the three phases and this reflects regulation by cytokine signaling and the phase-specific transcription factors. TCR, T-cell receptor; IL-7, interleukin-7.

GATA3 (Cismasiu et al. 2005; Tydell et al. 2007; Li et al. 2010b, 2013; Kueh et al. 2016). BCL11B is needed to finish the process of excluding cells from potential access to non-T-cell fates (Ikawa et al. 2010; Li et al. 2010a,b). It induces down-regulation of *Kit* expression, thus creating the DN2b phenotype, and direct binding of BCL11B at a possible *Kit* enhancer region may explain this ability to down-regulate *Kit* expression (H Hosokawa, HY Kueh, and EV Rothenberg, unpubl.). DN2b cells have a slower proliferation rate and begin to be desensitized to IL-7R signaling by a mechanism mediated by E proteins, and survival of DN2b cells becomes strictly Notch-dependent (Masuda et al. 2007; Wojciechowski et al. 2007; Yui et al. 2010). The subsequent DN3a stage is the peak period for expression of the Notch-dependent and E protein–dependent genes, such as recombination activating gene 1 (*Rag1*), *Rag2*, *Ptcra* (encoding pre-TCRα), and *Cd3e* (encoding CD3ε) (Takeuchi et al. 2001; Ikawa et al. 2006; Schwartz et al. 2006; Georgescu et al. 2008; Welinder et al. 2011). Therefore, DN3a cells are committed to T-cell fate even before expressing TCR on their surface, but are primed both for *TCRβ* (or *TCRγ* and *TCRδ*) gene rearrangement and for successful rearrangement to have an impact (Fig. 1).

Phase 3

The DN3a cells that have achieved successful V(D)J rearrangement for the *TCRβ* gene express pre-TCR and proceed to the DN3b stage. For DN2b and DN3a cells that rearrange both *TCRγ* and *TCRδ* productively, a separate pathway leads to development of several functionally distinct subsets of γδ T cells (Prinz et al.

2013; Vantourout and Hayday 2013). Although Notch signals are still required initially for passage through β-selection, cells that are able to receive signals through the pre-TCR transit from Notch-dependent to Notch-independent at this point. They strongly and rapidly turn off Notch target genes and IL-7R expression (Maillard et al. 2006; Taghon et al. 2006), becoming DN3b and then DN4 cells.

Signaling from pre-TCR triggers another shift in the gene regulatory network, transitioning into "phase 3." Despite the shutoff of IL-7R- and Notch-dependent growth-supporting systems, the DN4 cells enter a period of very rapid proliferation that is important for full phenotypic differentiation to the next stage (Kreslavsky et al. 2012). This is supported in part by chemokine signaling through CXCL12/CXCR4 (Janas et al. 2010; Tussiwand et al. 2011). The proliferative burst also appears to be supported by very dynamic use of TCF1 and LEF1 in what may be a transient, self-limited canonical Wnt pathway response (Yu et al. 2010), although the details are still under debate. In the process, the cells begin to express not only the CD4 and CD8 coreceptors but also two new transcription factors, IKZF3 (Aiolos) and RORγt, while finally silencing the last of the DN-specific factors, like ERG and HES1.

In summary, early T-cell development can be divided into three phases based on the status of T-lineage commitment and Notch dependency: Notch-dependent precommitted (phase 1), Notch-dependent T-lineage committed (phase 2), and Notch-independent pre-TCR-dependent (phase 3) phases (Yui and Rothenberg 2014). Each of the phases is associated with distinct transcription factor ensembles that are supported by extracellular signaling provided from the microenvironment in the thymus. All three phases are essential for proper αβTCR$^+$ T-cell development.

ROLES OF ENVIRONMENTAL SIGNALS AND TRANSCRIPTION FACTORS IN THE INITIATION OF T-CELL PROGRAM—PHASE1

In phase 1, early T cells need to rapidly proliferate to maintain the pool size of pro-T cells, because only small numbers of T-cell progenitors migrate into the thymus per day. The microenvironmental signals best established to support growth of initial thymic immigrants are Notch ligands and Kit ligand (also called stem-cell factor [SCF]). ETP cells have a NOTCH1$^+$ KIT^{hi} IL-7R^{low} phenotype initially and only later develop into NOTCH1$^+$ KIT^{hi} IL-7R^{hi} DN2a cells. Initially, the survival and proliferation of ETP cells mostly appear to be dependent on KIT/KIT ligand interaction (Waskow et al. 2002; Massa et al. 2006). Upon entry, thymic immigrants also express FLT3, but it seems to be down-regulated midway through the ETP stage, around the time when adult ETPs lose B-cell potential (Sambandam et al. 2005; Heinzel et al. 2007; Zhang et al. 2012; Mingueneau et al. 2013; Ramond et al. 2014). Although important prethymically, FLT3 does not appear to encounter its ligand in T-cell-promoting niches within the thymus. However, for cells escaping T-lineage commitment, FLT3 ligand may be important in much rarer, dendritic-cell-promoting intrathymic niches (Lyszkiewicz et al. 2015).

Notch1-DLL4 signaling is not only needed for viability and to induce genes encoding T-cell-specific regulatory factors, such as *Tcf7*, *Gata3*, and *Bcl11b*, but it is also required to antagonize alternative non-T-lineage development through a variety of pathways. A particularly sensitive target is the B-cell pathway (Mohtashami et al. 2010; Van de Walle et al. 2011), which may be blocked by Notch signaling through at least two distinct mechanisms (see below) (Hozumi et al. 2008). After this, ETP and DN2a cells still retain the potential to develop into dendritic cells, granulocytes, macrophages, and innate lymphocytes (ILCs), including NK cells and possibly mast cells (Shen et al. 2003; Schmitt et al. 2004; Balciunaite et al. 2005b; Taghon et al. 2007; Bell and Bhandoola 2008; Wada et al. 2008; Luc et al. 2012; Wong et al. 2012; De Obaldia et al. 2013a), but then all of these potentialities are also shut off at the transition from DN2a to DN2b under the continued influence of Notch signaling, as described below.

In view of their differential expression of KIT and IL-7R, it is notable that both ETP and

DN2a cells proliferate extensively before commitment, and both KIT ligand and IL-7 from the thymic microenvironment are crucial for this early expansion (Prockop and Petrie 2004; Massa et al. 2006; Calderon and Boehm 2012; Buono et al. 2016). This proliferation is driven first by a KITL-predominant microenvironment and then by an IL-7-predominant environment (Buono et al. 2016), matching the shifts in the ratio of KIT to IL-7R as the cells progress from ETP to DN2a cells. However, computational modeling suggests that the largest number of cell cycles may take place in the ETP stage (Manesso et al. 2013), before the cells activate strong expression of IL-7 receptors. This suggests that the earliest proliferative expansion could be a major function that depends not only on KIT signaling but also on another regulatory input in place of IL-7R, and one possibility is that this is supplied by the phase 1–specific transcription factors. Many of the transcription factors that are expressed only in phase 1 have roles in the proliferation, survival, and self-renewal of other hematopoietic cells and can be involved in the pathogenesis of leukemias (Yui and Rothenberg 2014).

Two phase 1–restricted transcription factor genes that have clear genetically defined roles in ETP-DN2-stage proliferative expansion are *Lyl1* (Zohren et al. 2012) and *Hhex* (Goodings et al. 2015; Jackson et al. 2015), whereas the factor encoded by a third gene, *Erg*, is specifically implicated in proliferation at the expense of differentiation (Knudsen et al. 2015). In addition, even PU.1 (encoded by *Spi1*, also known as *Sfpi1*), a phase 1–restricted transcription factor that seems most associated with alternative differentiation pathways, may also positively regulate growth of phase 1 pro-T cells, directly or indirectly. The expression of PU.1 continues in early DN cells from their prethymic precursors, persisting at high levels through multiple cell divisions in ETP and DN2a stages, but then decreasing sharply during T-cell lineage commitment (Yui et al. 2010). PU.1 is normally associated with the differentiation of dendritic cells (DCs), myeloid cells, and B cells, and its expression strongly correlates with the ability of uncommitted T-cell precursors to differentiate to DC and myeloid cell fates when Notch signaling is withdrawn (Lefebvre et al. 2005; Franco et al. 2006; Laiosa et al. 2006; Carotta et al. 2010b; Del Real and Rothenberg 2013). However, in addition, it is important for the expression of FLT3 by prethymic precursors (Carotta et al. 2010a), and direct genomic analysis indicates that it regulates numerous G-protein-coupled receptors and signaling components as well as cytokine receptors in phase 1 pro-T cells (Champhekar et al. 2015). Any of these could help to explain how PU.1 also positively contributes to early T-cell development (Dakic et al. 2005; Champhekar et al. 2015).

GATA3 AND THE ESTABLISHMENT OF A T-CELL-SPECIFIC REGULATORY STATE

Despite the presence of non-T lineage factors, phase 1 is also the context in which the T-cell program is first triggered. Notch signaling in ETP cells initiates the expression of the crucial regulatory transcription factor genes *Gata3* and *Tcf7*, which then alter the developmental status of the cells (Hosoya et al. 2009; Germar et al. 2011). *Tcf7* is activated by Notch signaling directly, and its product, TCF1, primarily acts as a positive regulator of T-cell specification, collaborating with Notch to activate T-cell genes through a feedforward network circuit (De Obaldia and Bhandoola 2015). GATA3 and TCF1 (encoded by *Tcf7*) not only begin to antagonize the progenitor-specific factors, but they also collaborate with a different group of regulatory factors required for T-cell development that continue expression initiated from a prethymic stage, such as MYB, RUNX1, CBFβ, IKAROS, GFI1, and E2A. These are stably expressed as "legacy factors," but nevertheless function in developmentally modulated ways, as they are incorporated together with TCF1 and GATA3 into the network of factors regulating T-cell lineage commitment. Actually, some of these factors have also been identified as physically GATA3-interacting molecules in an early T-cell line (H Hosokawa and EV Rothenberg, unpubl.).

GATA3 has indispensable roles not only in early T-cell development but also throughout

T-cell development, with multiple functions in the thymus and periphery. It works differently in a broad variety of developmental stages, affecting early T-cell survival, growth, specification, and commitment and in priming DN2 cells for progression to β-selection (Pai et al. 2003; Hosoya et al. 2010; Garcia-Ojeda et al. 2013; Scripture-Adams et al. 2014; Tindemans et al. 2014). In one of the earliest steps in commitment, Notch signaling- and TCF1-induced *Gata3* expression blocks B-cell lineage developmental potential from ETP cells, very shortly after their immigration into the thymus (Heinzel et al. 2007; Hozumi et al. 2008; Garcia-Ojeda et al. 2013).

The levels of GATA3 gradually increase in DN2a-DN2b cells and contribute to both activation of *Bcl11b* and repression of *Sfpi1* (PU.1) expression to support T-cell specification and commitment (Taghon et al. 2007; Zhang et al. 2012; Li et al. 2013). Knockdown of *Gata3* expression in ETP and DN2 cells blocks appearance of the DN3 phenotype, with up-regulation of phase 1 genes, including *Sfpi1* and *Bcl11a*, and down-regulation of phase 2 genes, such as *Ets1*, *Zfpm1*, *Il9r*, *Il17rb*, and *Bcl11b* (Garcia-Ojeda et al. 2013; Scripture-Adams et al. 2014). Later, GATA3 is also required for full TCRβ locus activation needed to pass through β-selection, and for generation of CD4 SP T cells (Pai et al. 2003).

Therefore, GATA3 is a vitally important factor for early T-cell development, but at least in the murine system, it has a highly limited dose–response range in DN cells. This is an interesting contrast with its roles in mature Th2 cells, where overexpression is tolerated. Instead, increased concentrations of GATA3 are as toxic for mouse early T-cell precursors as loss of GATA3, and if the overexpressing cells can be kept alive, they can be driven into becoming mast cells (Taghon et al. 2007). Thus, T-cell identity is strongly dependent on GATA3 and also requires the preservation of strict limits to GATA3 expression.

While GATA3 at modest levels is nearly universal in T-lineage precursor cells from the ETP stage onward, its activity may be modulated in a stage- or signal-dependent way. Recently, several groups reported that posttranslational modifications of GATA3 regulate its functions (Cook and Miller 2010; Kitagawa et al. 2014; Hosokawa et al. 2015, 2016). Phosphorylation and acetylation regulate both stability and transcriptional activity of the related GATA1 factor (Hernandez-Hernandez et al. 2006). Thus, the functions of GATA3 could be strictly regulated by several distinct mechanisms in a stage-specific manner.

AVOIDING DIVERSION TO ALTERNATIVE FATES IN PHASE 1

Phase 1 cells, still being uncommitted, need continuous signaling from the microenvironment to stay in the T-cell pathway. Each kind of alternative pathway needs to be obstructed by a different mechanism. Within the thymus, Notch-DLL4 signaling rapidly induces antagonists of at least two alternative pathways, GATA3 to block B-cell development and HES1 to block myeloid development (De Obaldia et al. 2013b; Garcia-Ojeda et al. 2013; Scripture-Adams et al. 2014). Notch-induced TCF1 may also play a role in blocking access to DC development, at least (Kueh et al. 2016). Eventually, the cumulative action of the Notch-triggered regulatory cascade also induces BCL11B (Kueh et al. 2016) to block NK cell development (Li et al. 2010b). The up-regulation of these active antagonists of alternative fates can be viewed as "commitment by addition." The possibility of diversion to myeloid and dendritic cell fates is not eliminated at a cell-intrinsic level, however, until the cells transition to phase 2, through "commitment by subtraction," when the key enabling factor PU.1 is silenced. Until then, very selective expression of cytokines in the thymus microenvironment also provides a safety net. B cells can potentially grow in the IL-7 provided by cortical thymic epithelial cells (Calderon and Boehm 2012), if not aborted early by Notch-dependent mechanisms, but progenitors taking a pathway that might require myeloid growth factors cannot expand in the normal thymus where such factors are largely absent (Lyszkiewicz et al. 2015). In fact, these environmental restrictions are extremely effective, so that the majority of phase 1 cells appear not to give rise to any de-

tectable myeloid progeny in vivo as long as their development in the thymus is undisturbed (Schlenner et al. 2010).

Other cytokines besides myeloid growth factors must also be kept away from developing pro-T cells. A fraction of phase 1 cells can respond to the addition of cytokines IL-33 and IL-7 in the presence of Notch signaling to become ILC2 (Wong et al. 2012). Signals from IL-33 can redirect the effects of transcription factors such as GATA3, BCL11B, and TCF1 to work in the ILC2 program, via induction of *Id2* and the gene that encodes orphan nuclear receptor RORα (Hoyler et al. 2012; Wong et al. 2012; Yang et al. 2013; Walker et al. 2015; Yu et al. 2015; Zhong and Zhu 2015). *Id2* expression is required to develop all types of ILCs, which do not use RAG-based gene rearrangement in their development program. ILCs keep the *Rag* genes off, at least in part because of neutralization of E protein activity by Id2 (Diefenbach et al. 2014; De Obaldia and Bhandoola 2015; Serafini et al. 2015). In contrast, high levels of BCL11B, exceeding those in any ILC subset, restrain *Id2* expression in cells taking the T-cell pathway (Longabaugh et al. 2017).

PROLIFERATION AND DIFFERENTIATION IN THE PHASE 1/PHASE 2 TRANSITION: ROLES OF IL-7/IL-7R SIGNALS

IL-7 plays a key role in the development of early T cells. Actually, mice lacking IL-7 or IL-7R show strongly impaired T-cell development (Peschon et al. 1994; DiSanto et al. 1995; von Freeden-Jeffry et al. 1995), and IL-7R-deficient T-cell precursors cannot fill available thymic niches (Prockop and Petrie 2004; Zietara et al. 2015). Defects in the IL-7R in human are associated with a severe combined immunodeficiency syndrome characterized by a complete lack of T cells (Noguchi et al. 1993; Puel et al. 1998). IL-7 signaling also has critical roles in both γδ T-cell development and in later CD8 lineage choice via induction of transcription factor Runx3 (Singer et al. 2008).

IL-7R is strongly up-regulated in DN2a, and the proliferative expansion of the cells at this stage becomes highly sensitive to the IL-7 level (Wang et al. 2006). In addition, however, its signaling modulates the rate of developmental progression (Balciunaite et al. 2005a; Huang et al. 2005), and this implies an interaction with the T-cell specification gene-regulatory network. IL-7R signaling activates both signal transducers and activators of transcription (STAT)5 and phosphatidylinositol 3-kinase (PI3K) pathways. Both pathways are important: the PI3K pathway plays a crucial role in proliferation and survival of early T cells (Pallard et al. 1999) and STAT5A/5B are needed for full population expansion both before and after β-selection (Yao et al. 2006), as well as being indispensable for opening of the *Tcrg* loci for rearrangement to allow γδ T-cell development (Ye et al. 2001; Kang et al. 2004). Surprisingly, though, little is known about any other ways that STAT5A/5B interact with the genes in the T-cell specification gene network.

Two unresolved questions are: first, whether IL-7R contributes to the extensive proliferation that seems to occur in ETPs before IL-7R expression is detectable; and, second, why KIT and IL-7R signaling activate less rapid proliferation in DN2b cells than in DN2a cells despite similar expression of IL-7R. One report has suggested that KIT and IL-7R can interact physically and signal as a complex (Jahn et al. 2007). If responses to IL-7R are actually responses to an IL-7R/KIT complex, then a high level of KIT coexpression, only available in phase 1 cells, may be required to support full IL-7-mediated proliferation. Alternatively, IL-7R signaling may depend on phase 1 transcriptional regulatory factors to induce proliferation of early T cells.

Desensitization of IL-7R after the phase 1–phase 2 transition may be important in part because of cross talk of IL-7 signaling with transcription factors. There are positive and negative interactions of IL-7/IL-7R signals with key T-cell transcription factors. First, IL-7R-mediated PI3K/AKT signaling has been reported to induce phosphorylation of GATA3 in memory Th2 cells (Hosokawa et al. 2016), and AKT-mediated phosphorylation of GATA3 regulates organization of GATA3 complexes. Strict regulation of GATA3 expression and function is important for survival and proliferation of

early T cells; thus, IL-7R-mediated phosphorylation of GATA3 may have some role in stage-specific responses to IL-7. Second, IL-7/IL-7R signaling is known in some conditions to reduce expression of genes, including *Tcf7*, *Lef1*, and *Bcl11b*, encoding some phase 2 transcription factors that have important roles in the phase 2 regulatory network (Yu et al. 2004; Ikawa et al. 2010). This interaction may underlie the ability of high IL-7 to slow developmental progression to the DP stage, especially in cells developing from adult ETPs and bone marrow precursors (Balciunaite et al. 2005a; Huang et al. 2005).

Despite its relative desensitization during DN2b and DN3a stages, IL-7R activity remains a latent contributor to cell survival as long as *Il7r* is expressed. As cells enter β-selection, IL-7R signaling from existing cell-surface receptors still provides some support for proliferation and survival of DN3b and DN4 cells, even as transcription of *Il7r* itself is finally repressed. At this stage, IL-7/IL-7R signaling enhances survival and proliferative expansion via induction of prosurvival factor *Bcl2* and by sustaining expression of nutrient-transport protein-coding genes, such as *Cd98* and *Cd71* (Boudil et al. 2015). Therefore, sensitivity and reactivity to IL-7 are dramatically changed in three phases.

ROLES OF CYTOKINES AND TRANSCRIPTION FACTORS IN THE COMMITMENT OF T-CELL LINEAGE CELLS—PHASE 2

Right after *Bcl11b* expression is induced by the combination of Notch signaling, RUNX1, GATA3, and TCF1 cells transition to the DN2b stage (Yui et al. 2010; Li et al. 2013; Kueh et al. 2016). DN2b cells proliferate more slowly than DN2a, and their survival becomes strictly Notch-dependent (Yui et al. 2010). It is still not certain whether BCL11B directly represses most phase 1 regulatory genes or simply disables signaling that would otherwise maintain their expression (Longabaugh et al. 2017); but in either case, its action allows most phase 1–restricted genes to be silenced and enables the cells to enter a committed DN2b state (Ikawa et al. 2010; Li et al. 2010a,b).

As DN2a cells progress into the DN2b stage, they undergo dynamic shifts in the expression of other transcription factors as well. These are currently useful landmarks although their functional significance is still not fully understood. Some of the largest shifts are reciprocal regulation of members of the same factor family. For example, two ETS family transcription factor genes, *Ets1* and *Ets2*, are rapidly turned on as the ETS family gene *Sfpi1* is turned off; *Gfi1* is turned on as *Gfi1b* is turned off; *Bcl11b* is turned on as *Bcl11a* is turned off, and *Runx1* increases expression as *Runx2* and *Runx3* decrease (David-Fung et al. 2009). The silencing of *Sfpi1* expression, through a mechanism dependent on RUNX factors and possibly GATA3 (Taghon et al. 2007; Huang et al. 2008; Zarnegar et al. 2010; Scripture-Adams et al. 2014), is thought to be one of the molecular mechanisms of alternative lineage exclusion as noted above.

Several mechanisms can amplify Notch pathway signaling in DN3a cells. In addition to *Notch1*, *Notch3* is activated at DN3a, largely by a NOTCH1-derived signal, and this probably adds to the sensitivity for Notch pathway signaling. At the same time, the cells become desensitized to IL-7R-driven mitogenesis in a process that depends on the basic helix–loop–helix E protein transcription factors, E2A and HEB (Wojciechowski et al. 2007). Whereas E2A (encoded by *Tcf3*) is expressed stably, HEB levels (encoded by *Tcf12*) increase steadily through the DN stages so that an increasing amount of E2A/HEB heterodimer can be formed, which is important for β-selection (Barndt et al. 2000; Braunstein and Anderson 2012). The activity of E2A and HEB may be further boosted in DN3a by the silencing of a competitive binding partner, *Lyl1*, one of the phase 1 genes (Zhong et al. 2007; Yui et al. 2010). E proteins in T-cell precursors work in collaboration with Notch signals (Ikawa et al. 2006), not antagonistically as proposed in B cells (Nie et al. 2008), and Notch signaling levels can only be sustained in this context by strong E protein activity (Yashiro-Ohtani et al. 2009; Del Real and Rothenberg 2013). Expression of E protein–dependent and Notch-dependent genes, including *Ptcra*, *Rag1*, *Rag2*, terminal de-

oxynucleotidyl transferase (*Dntt*) and *Cd3e*, markedly increases as the proliferation slows and the cells proceed to the DN3a stage (Takeuchi et al. 2001; Ikawa et al. 2006; Schwartz et al. 2006; Georgescu et al. 2008; Welinder et al. 2011; Xu et al. 2013).

The E proteins also activate expression of growth-inhibitory factors, such as suppressor of cytokine signaling 1 (*Socs1*) and *Socs3*, which uncouple growth factor receptors like IL-7R from their signaling pathways. In parallel, they can directly inhibit cell-cycle activation genes, and induce cyclin-dependent kinase inhibitors (Schwartz et al. 2006). The cell-cycle arrest induced by E proteins is important to enable RAG1/RAG2-mediated recombination of the TCR genes to take place (Li et al. 1996) as well as for checkpoint control. Double knockout of the genes encoding E2A (*Tcf3*) and HEB (*Tcf12*) in DN3 stage not only prevents proliferative arrest but also enables DN3 cells to undergo reverse differentiation to return to a highly IL-7-responsive, DN2-cell-like phenotype (Wojciechowski et al. 2007). However, E protein effects on TCR expression go beyond their effects on the cell cycle. Singly, E2A (*Tcf3*)-deficient cells have a specific defect in *TCRβ* gene rearrangement (Agata et al. 2007) and are prone to leukemic transformation (Engel and Murre 2004).

To summarize, DN3a cells generated by phase 2 regulatory processes are committed to a T-cell fate that is αβ lineage biased and primed for RAG-mediated *TCRβ* gene rearrangement. To reach this stage, cells require Notch signaling, GATA3, TCF1, BCL11B, ETS1, RUNX1, and E2A (Oosterwegel et al. 1991; Schwartz et al. 2006; Li et al. 2010a,b; Germar et al. 2011; Zhang et al. 2012; Xu et al. 2013). The maximal expression of the *Cd3* gene cluster is regulated by E protein, GATA3, TCF1, and BCL11B, whereas the *Rag* genes are induced by E protein and GATA3. Together with Notch signaling, several transcription factors are involved in *TCRβ* gene rearrangement such as E2A, HEB, MYB, GATA3, and RUNX1. ETS1 collaborates with RUNX1 to activate the enhancer of the *TCRβ* gene (Gu et al. 2000). The expressions of *Hes1*, *Notch3*, and *Ptcra* are jointly regulated by E protein and Notch signaling. E proteins are required to promote and sustain NOTCH1 expression in DN3a stage, and Notch signaling can be antagonized by the E protein antagonist Id2. BCL11B at a high level keeps *Id2* expression silent and assists activation of *Dntt* and the *Cd3* gene cluster (Longabaugh et al. 2017) to support transition to the DN3a state. Thus, most of the T-cell genes are fully activated, and cell-cycle arrest is induced, by late phase 2 stage. At this stage, RAG1/RAG2-mediated programmed *TCRβ* (V(D)Jβ) gene rearrangement also takes place. In the mouse, only a minority of phase 2 cells rearrange the *Tcrg* and *Tcrd* genes instead. The cells expressing a functional pre-TCR on productive rearrangement of the *Tcrb* gene are able to pass through the β-selection checkpoint.

ROLES OF TRANSCRIPTION FACTORS IN PASSING THE β-SELECTION CHECKPOINT TO PHASE 3

Signaling through the newly expressed pre-TCR not only disrupts the quiescence of DN3a cells, but also rapidly shuts off the expression of Notch target genes in an IKAROS-dependent manner, and cells proceed to phase 3 (Chari and Winandy 2008; Kleinmann et al. 2008; Geimer Le Lay et al. 2014). The burst of proliferation that takes place on β-selection amplifies clones of cells with productive *Tcrb* rearrangements to maximize the chance for productive TCRαβ diversity. At the same time, it helps αβ T cells to undergo proliferation-dependent epigenetic changes and to dilute out previous stores of regulatory molecules that would otherwise interfere with their new regulatory state (Kreslavsky et al. 2012). Notch signal–dependent transcription becomes dispensable, and many of the transcription factors that participated in the phase 1 and phase 2 stages decrease or disappear (Tabrizifard et al. 2004; Yui and Rothenberg 2004). As cells become DP thymocytes, repressive histone marks accumulate at the promoters of phase 1 and Notch target genes (Zhang et al. 2012; Vigano et al. 2014), whereas other sites open (e.g., a set of DP-specific sites for ETS1) (Cauchy et al. 2015). The establishment of durable epigenetic changes at

the T-cell and non-T-cell gene loci makes the program of T-cell gene expression sustained and irreversible (Zhang et al. 2012; Vigano et al. 2014).

As the cells proliferate and become DP, in response to a combination of TCF1 and β-catenin, with a required input from MYB, the cells distinctively activate expression of two new factors: RORγt (*Rorc*) and the Ikaros zinc-finger family member AIOLOS (*Ikzf3*). Although most universal T-cell properties have already been acquired by the cells, they need a special form of viability support to keep them alive in DP stage long enough to undergo several rounds of TCRα gene rearrangement. The combination of MYB, TCF1, high HEB/E2A, and RORγt helps to induce BCL-XL (*Bcl2l1*) expression in the future DP cells to sustain them through this process (Sun et al. 2000; D'Cruz et al. 2010; Yuan et al. 2010; Wang et al. 2011) and to arm them for positive selection. Thus, a new regulatory state prepares DP thymocytes for the complex positive and negative selection events that they must undergo.

REGULATING T-CELL PRECURSOR FLUX: GLIMPSES INTO THE EARLIEST PORT OF THYMOCYTE ENTRY

One domain in which microenvironmental cues may be most important for controlling T-cell generation remains relatively mysterious. This is the initial entry port for immigrants into the thymus. Early T-cell development is coordinated with migration through distinct thymic microenvironments (Fig. 2) (Petrie and Zuniga-Pflucker 2007; Love and Bhandoola 2011). In postnatal mammals, immigrant precursors initially enter the thymus through blood vessels near the corticomedullary junction (Fig. 2, right), drawn possibly by chemokine receptor signaling from CCR7 and CCR9 (Uehara et al. 2002; Schwarz et al. 2007; Krueger et al. 2010; Zlotoff et al. 2010). Although the numbers of cells involved are small, it appears that ETP cells undergo expansion with minimal differentiation in this corticomedullary junction region, and then, days later, differentiate into DN2a cells that can begin their migration from the site of entry deep within the cortex to the outer rim of the cortex. β-selection occurs during the accumulation of the DN3 cells when they reach the extreme outer portion of the thymus. A directional reversal of migration back across the cortex toward the medulla occurs for the later stages of thymocyte development. Thus, for example, the different requirements for IL-7 may be accommodated in vivo by varying amounts of IL-7 in combination with other extracellular signals, including adhesive molecules, chemokines, and cytokines provided by different microenvironments (Zamisch et al. 2005; Alves et al. 2009; Griffith et al. 2009; Love and Bhandoola 2011).

This pattern is different in the early fetal thymus, where precursors enter directly into the thymic rudiment by migrating across the mesenchyme from the outside, before the organ is vascularized and before the capsule forms a barrier (Fig. 2) (Anderson et al. 2006). In the fetus, elegant mutant-rescue experiments indicate that the crucial genes that the epithelium must express to support T-cell differentiation are DLL4, KIT ligand (KITL), CXCL12, and CCL25 (i.e., ligands for NOTCH1, KIT, CXCR4, and CCR9, respectively) (Calderon and Boehm 2012). However, it is not clear that this fetal environment behaves equivalently to the postnatal one. First-wave fetal thymocytes differentiate faster than late-fetal or postnatal thymocytes (Watanabe et al. 1997; Ramond et al. 2014; Scripture-Adams et al. 2014), and they begin to carry out TCR gene rearrangement after fewer rounds of proliferation once they have entered the thymus (Lu et al. 2005). At least part of this difference is cell-intrinsic, because fetal thymocytes also differentiate faster when they are compared on the "level playing field" of the OP9-DLL1 artificial stroma coculture system (Schmitt and Zuniga-Pflucker 2002), and there are subtle but real differences in gene expression at corresponding developmental stages (David-Fung et al. 2006; Belyaev et al. 2012; Ramond et al. 2014; Scripture-Adams et al. 2014). First-wave fetal thymocytes also rearrange a particular subset of TCRγ genes that are almost never used after birth. However, in the adult thymus, the specific transition from

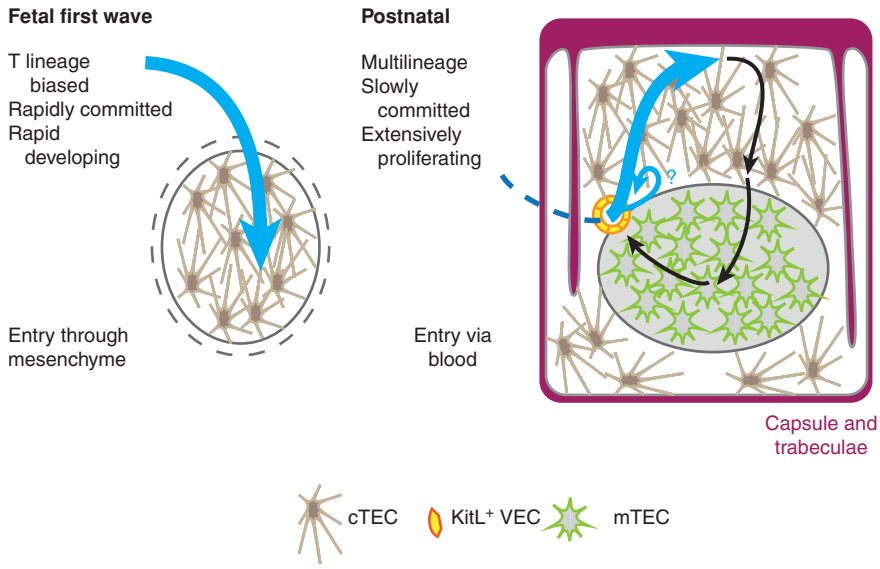

Figure 2. Fetal and postnatal pathways for progenitor entry into the thymus. Thymic epithelial function is established in embryonic life and maintained through postnatal life by genes controlled by the FOXN1 transcription factor, but the interactions between the thymic microenvironment and immigrating T-cell precursors are not the same in fetal and adult life. (*Left*) Schematic showing pathway of entry and cells involved in interactions of initial wave of T-cell precursors with the E12-14 fetal mouse thymic microenvironment. (*Right*) Same for pathways and interactions between postnatal T-cell precursors and the young adult (4–8 wk old) mouse thymus. Emphasis is on the cortex where progenitors undergo T-lineage commitment and the interactions controlling traffic into this domain. Medullary structure (pale green, *right* panel only) develops after the entry of the first-wave fetal thymic immigrants. Cyan arrows: major pathways of entering precursors, through T-cell lineage commitment. A thin cyan arrow indicates possible self-renewal pathway within entry compartment (*right*). Thin black arrows represent locations of T-cell receptor (TCR)-dependent phases of T-cell development in the postnatal thymus (*right*): β-selection, positive selection in the cortex leading to migration into the medulla, and medullary maturation events including selection and preparation for export. Locations of cortical thymic epithelial cells (cTEC), KITL$^+$ vascular endothelial cells (KITL$^+$VEC), medullary thymic epithelial cells (mTEC), and the connective tissue elements of the thymus (capsule and trabeculae, *right* panel only) are indicated. See text for details.

ETP to DN2 is disproportionately slower than in the fetus. In steady state, postnatal mouse ETPs take at least 7–10 days before they shift to the DN2a stage, although progression to subsequent stages may require only ~2 days per step (Porritt et al. 2003; Manesso et al. 2013). By contrast, the ETP to DN2a transition takes no more than 1–2 days in the fetus. This postnatal-specific extension of the ETP period is poorly reproduced in the OP9-DLL1 or OP9-DLL4 culture system, where most of the extended proliferation takes place in the DN2a/2b stages. In fact, one of the differences between "ETP" cells growing on OP9-DLL1 and those harvested ex vivo is the weaker expression of FLT3, KIT, and several other stem or progenitor-cell regulatory genes in vitro that are normally ETP specific in vivo (Zhang et al. 2012; Mingueneau et al. 2013). What is clearly missing in the OP9-DLL1 culture is an equivalent of the entry compartment. Thus, it is exciting that there is evidence that particular niches in the adult thymic microenvironment not only limit the carrying capacity of the thymus (Zietara et al. 2015) but also preserve the immature ETP status.

Normally, all entering cells differentiate or die: self-renewing stem cells do not persist in the initial entry compartment. However, two groups showed recently that the niche itself can support extremely prolonged self-renewal

in cases where there is no competition from new waves of immigrating precursors from the bone marrow (Martins et al. 2012; Peaudecerf et al. 2012). Thus, it is possible that the initial entry compartment, the ETP niche, intrinsically supports expansion and delays differentiation. If this is true, then it provides something distinctive other than a Notch ligand. The recent definition of markers that distinguish the cells forming these initial entry niches (Buono et al. 2016) can help greatly to characterize what these self-renewal cues may be.

Recently, cells expressing the membrane-bound form of Kit ligand (mKITL) were reported in a specific subdomain of the thymus, which may represent the entry port for thymus-seeding cells (Buono et al. 2016). Membrane-bound KITL is known to be more potent than secreted KITL, similar to other surface-tethered cytokines in the bone marrow microenvironment. Surprisingly, a subset of vascular endothelial cells (VECs) in the cortex specifically expresses both DLL4 Notch ligands and mKITL, and they act as a specific niche for ETP cells. VEC-specific deletion of the *KitL* gene results in a significant decrease of the number of ETP cells in the thymus. DN2a cells, on the other hand, are supported by IL-7-expressing cortical thymic epithelial cells (cTECs), which also express DLL4 but at lower levels than on the VECs, and express a higher fraction of KITL in the less-potent soluble form (Buono et al. 2016). Both the $KITL^+$ VECs and the cTECs express one important lymphocyte-supporting chemokine, CXCL12 (ligand for the CXCR4 chemokine receptor), but the cTECs express much more CCL25 (ligand for the CCR9 chemokine receptor) (Buono et al. 2016), which is also important for T-cell development. These results suggest that early T cells migrate inside of the thymus through stage-specific niches that may provide them with differentially optimized regimes of extracellular signaling. Specific niches for DN2b, DN3, and DN4 have not been reported yet, but it is assumed that there are some niches to support their proliferation, survival, and construction of stage-specific gene regulatory networks.

Importantly, lacking the vascular endothelial entry compartment, the initial port of entry in the fetal thymus appears to be the immature cortical thymic epithelium itself. Thus, the initial compartment in which the ETP to DN2 transition occurs is different in the adult and fetal thymuses. One interesting possibility is that the lack of a VEC entry compartment itself promotes the rapid kinetics of fetal T-cell differentiation. This speculation remains to be tested, but one possibility is that signals provided by the adult entry niche actually retard dif-

Table 1. Expression of cytokine receptors in early T-cell precursors

	ETP	DN2a	DN2b	DN3a	DN3b	DN4
Flt3	High→low	Neg	Neg	Neg	Neg	Neg
Kit	High	High	Mid	Low	Neg	Neg
IL-7R	Low	High	High	High	High	Neg
IL-4R	High	High	High	Low	Low	Mid
IL-9R	Low (mid in fetal)	Low (mid in fetal)	Low (mid in fetal)	Low	Low	Mid
PDGFRβ	High	High	Mid	Mid	Low	Low
γ_c (*Il2rg*)	High	High	High	High	High	High
IL-2Rα (CD25)	Low	High*	High*	High*	High→mid*	Neg
IL-2Rβ	Neg	Neg	Neg	Neg	Neg	Neg
PDGFRα	Neg	Neg	Neg	Neg	Neg	Neg

Data are based on RNA-seq (Zhang et al. 2012) and microarray (Mingueneau et al. 2013) measurements of messenger RNAs (mRNAs) encoding the indicated receptors. Data for early thymic progenitor (ETP) and DN2 subsets derived from fetal liver precursors were from (Zhang et al. 2012). The γc chain (CD132, encoded by *Il2rg*) is the common heterodimer partner of the interleukin-7 receptor (IL-7R)α, IL-4Rα, and IL-9Rα chains. Regarding IL-2Rα, asterisks indicate that these early T-lineage cells do not express functional IL-2 receptors even when they express high γc and IL-2Rα, because they do not express IL-2Rβ, which is a required functional component of both the IL-2R and the IL-15R. Neg: Negative (<1 fpkm).

ferentiation to enable more extensive proliferation, and that transition out of a potentially self-renewing early-ETP regulatory state depends on release from this compartment.

The nature of such signals is still far from clear. One approach is via analysis of receptors expressed by the ETPs themselves. Based on our RNA-seq data, we found that ETPs express *Il4ra*, *Pdgfrb*, and possibly *Il9r* (Table 1), receptors whose roles in early T-cell development have not been clarified yet (Zhang et al. 2012). Recent RNA-seq data suggest that the two niches for phase 1 cells, VEC and mTEC, differentially express Pdgf family ligands (Fig. 3). Whether or

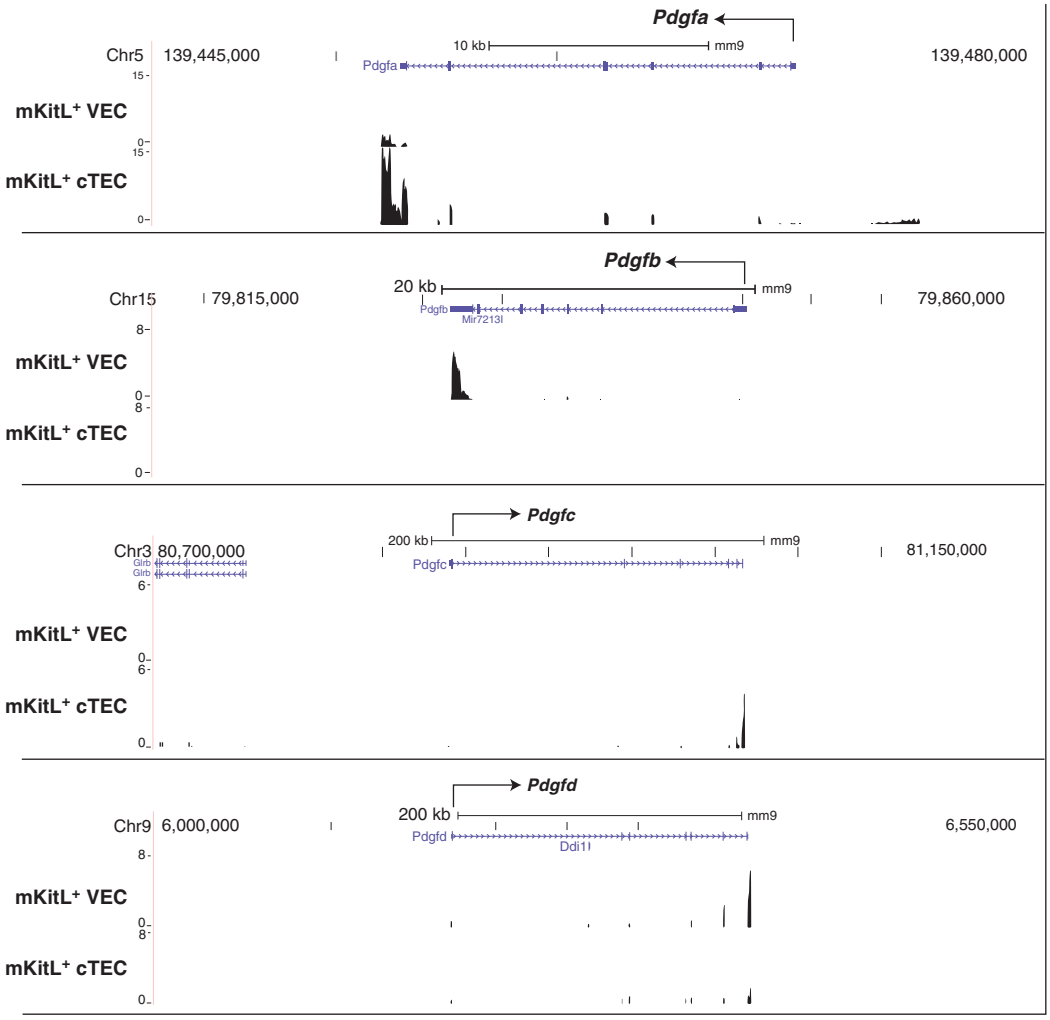

Figure 3. High specialization of different intrathymic phase 1 niches in postnatal mice: distinct gene expression patterns of Pdgf family ligands in niche cells for early entrants and differentiating phase 1 cells. RNA-sequencing tracks for the membrane-bound form of Kit ligand (mKITL)-expressing vascular endothelial cells (VECs) (early thymic progenitor [ETP] niche), and cortical thymic epithelial cells (cTECs) (DN2a niche) (Buono et al. 2016) were mapped against the murine (mm9) genome (National Center for Biotechnology Information [NCBI] build 37) on the University of California, Santa Cruz (UCSC) genome browser. Expression patterns of platelet-derived growth factor (PDGF) family ligands are shown. PDGF-B and -D RNAs were strongly detected in mKITL-expressing VEC cells, and PDGF-A and -C were preferentially expressed in mKITL-expressing cTEC.

not these particular ligand–receptor pairs control the starting gate for T-cell development, they offer fascinating hypotheses for exploration (H Hosokawa, M Yui, and EV Rothenberg, unpubl.).

CONCLUDING REMARKS

Early T-cell development is divided into three phases, based on Notch dependency and status of T-cell commitment, and all of the three phases are essential for proper T-cell development in the thymus. Inside of the thymus, pro-T cells undergo programmed migration and are supported by stage-specific microenvironments, which at a minimum provide Notch ligands and cytokine signaling. It is clear that stage-specific microenvironments are involved in the establishment of the stage-specific gene regulatory network. However, a great deal is yet to be explained and understood. Why is the effect of IL-7 on proliferative expansion of early T cells different in distinct stages? How are stage-specific transcription factors induced by extracellular signaling? And how are stage-specific roles of stably expressed transcription factors like GATA3 regulated by extracellular signaling? Are there any specific niches for DN2b, DN3, and DN4 cells as well as, possibly, for ETPs? What are the novel molecules provided by niches that support and regulate early T-cell development?

T-cell development requires a specific organ, the thymus, which provides well-regulated stage-specific microenvironments to T-cell progenitor cells. To understand T-cell development in perspective, it is important to address more deeply the molecular basis of cross talk between the extracellular signaling from the microenvironment and the cell-intrinsic gene regulatory networks in pro-T cells.

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